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(54) Title: TRANSGENIC PLANTS WITH REDUCED EXPRESSION OF ENDOGENOUS PHYTOCHROME A, EXHIBITING IMPROVED ROOTING, AND METHODS FOR THEIR PRODUCTION

(57) Abstract: A method for improving the rooting capacity of plants by downregulating the phytochrome A is disclosed, as well as transgenic plants having improved rooting capacity. Also the overall survival after potting capacity is improved in the plants produced according to this method.

Transgenic plants with reduced expression of endogenous phytochrome A, exhibiting improved rooting, and methods for their production

The present invention concerns transgenic plants and in particular transgenic plants exhibiting improved rooting capacity and/or improved survival after potting, and methods for their production.

Background of the invention

The importance to be able to propagate non-reproductive plant material can not be exaggerated, as successful propagation both *in vitro* or by cuttings of mature plant material can make it possible to speed-up the multiplying of important genetic plant material. Many commercially important plants, such as tree species, are very difficult to propagate both *in vitro* and by cuttings. The latter as a result of the fact that mature trees loose their potential of forming adventitious roots. Examples of tree species that are difficult to root are e.g. aspen, eucalyptus, spruces, birches and oaks. Various chemical compositions, such as growth mediums, gels and pastes for inducing improved rooting have been suggested. One possibility to improve the rooting capacity is by using biotechnological methods. This would be more useful, and possess considerable practical and commercial importance.

Phytochromes are a group of photoreceptors that detect light in the red and far-red wavelengths, and are involved in many developmental processes, such as seed germination, hypocotyl regulation, flowering and photoperiodism. The phytochromes are encoded by a multigene family. In the following description, this nomenclature will be adhered to: *PHYA* is the gene, PHYA is the apo-protein, phyA is the holoprotein. In *Arabidopsis*, five different phytochromes exist, phytochromes (PHY) A through E, in some cases with overlapping functions. Phytochrome A (phyA) is the only light labile phytochrome and has been shown to be the major receptor in for example, the classical very-low-fluence response (VLFR) and far-red high irradiance response (FR-HIR). PhyA is also an important detector of daylength, especially in long-day plants (see below).

Daylength regulates many different aspects of plant growth and development, including the induction of flowering, tuberisation, vegetative growth and bud dormancy. In many woody species growing in high latitudes, the initiation of cold acclimation and dormancy are synchronised with the end of the growth season and the onset of low temperatures in the

autumn. Long days (LDs) sustain shoot elongation, whereas short days (SDs) induce growth cessation, formation of terminal buds, cold acclimation and bud dormancy. In many temperate tree species photoperiodic ecotypes have been described, that differ in their responses to daylength and adaptation to various photoperiodic regimes.

Photoperiodism requires a means of monitoring time, and is thus dependent on an internal clock to perceive time and a photoreceptor to distinguish between light and dark. Our understanding of the circadian clock mechanism is still poor, but the recent identification and cloning of mutants aberrant in clock functions of the fly *Drosophila melanogaster*, the fungus *Neurospora crassa* and the plant *Arabidopsis thaliana* have given important insights into some of its functions. Research has shown that the photoreceptors phytochrome and cryptochrome have dual roles in the detection of daylength. Firstly, by mediating the light on and light off signals of dawn and dusk they entrain the circadian oscillator. Secondly, they gate many rhythmic responses.

Day-length perception can also be changed in plants by altering the levels of the photoreceptors by overexpressing or reducing the expression (by mutation or antisense transformation) of photoreceptor genes. Overexpression of *Arabidopsis PHYB*, or oat *PHYA* in *Arabidopsis* green seedlings has been shown to shorten the period of the circadian oscillations of *cab::luc* expression compared to the patterns in plants with wild type. Overexpression of these genes also results in earlier flowering under SD conditions than in WT plants. Conversely, the *phyA* mutation in *Arabidopsis* affects the plant's sensitivity to daylength perception, making it less sensitive to night breaks, which promote flowering in WT plants. In the one study, the WT flowered about six days earlier, and the mutant only two days earlier, with night breaks than without them. Without night breaks the two genotypes flowered at the same time. The *phyA* mutant of pea, *fun 1*, has also been shown to be insensitive to daylength extensions, giving altered flower properties and delayed flowering. PhyA has also been suggested to promote flowering in pea by reducing synthesis or transport of a flowering inhibitor. These examples show that phyA may have a role in the photoperiodic control of flowering in the long day plants (LDPs) *Arabidopsis* and pea. However, the means by which phyA is coupled to the circadian clock is still not clear.

Prior art

Phytochromes have besides the function in leaf and internode tissues also been shown to have a functional role in root tissue in tobacco plants (Adam, E., Szell, M., Szekeres, M., Schaefer, E. and Nagy, F. (1994). The developmental and tissue-specific expression of tobacco phytochrome A genes. *Plant J.* **6**, 283-293).

The present inventors have earlier shown that expressing the oat *PHYA* in the deciduous tree hybrid aspen (*Populus tremula* L. x *P. tremuloides* Michx.) results in plants that are severely dwarfed and insensitive to the induction of dormancy by SDs (Olsen, J. E., Junttila, O., Nilsen, J., Eriksson, M. E., Martinussen, I., Olsson, O., Sandberg, G. and Moritz, T. (1997). Ectopic expression of oat phytochrome A in hybrid aspen changes critical daylength for growth and prevents cold acclimation. *Plant J.* **12**, 1339-1350). This indicates that the phyA has importance in the process of shoot growth, but it can also be that phyA can function as an allocator of "energy" from shoot growth to root growth, i.e. the concept shoot/root growth. No effects on rooting capacity were however detected in this study.

WO 96/01897 discloses polynucleotide sequences complementary to the photoreceptor gene HY4 used in order to produce transgenic plants having shorter or longer stem lengths than substantially homozygous non-transgenic plants. One aim of the invention according to WO 96/01897 is to produce plants which are insensitive to blue light regulation of their growth and which may thus grow taller than wild type plants in regions with less than optimal sunlight. Another aim is to breed "dwarf" varieties of various plants, as shorter plants do not fall over ass easily as taller plants upon application of large amounts of fertiliser. However, the particular cultivar of tobacco used in the experiments disclosed in WO 96/01897 did not yield an adult "dwarf" phenotype, a result which gives an indication of the uncertainties involved in this technical field.

Heyer A. G. et al. (Function of Phytochrome A in Potato Plants as Revealed through the Study if Transgenic Plants, Plant Physiol. (1995) 109:53-61) discloses transgenic potato plants (*Solanum tuberosum*) containing the potato phytochrome protein encoded by the *PHYA* gene cDNA (phyA) in sense or antisense orientation.

Heyer A. and Gatz, C. (Plant Molecular Biology, 18:535-544, 1992) have isolated and sequenced a cDNA clone encoding the apoprotein of potato phytochrome and deduced the amino acid sequence. The sequence was found to show 78 % amino acid identity to

Arabidopsis phyA and 50 % identity to the *Arabidopsis* phyB ORF, supporting the classification of the cDNA clone as potato phyA phytochrome.

Summary of the invention

The present inventors have surprisingly found that that phyA is an important factor both in detection of daylength and rooting capacity and proven this concept in hybrid aspen by studying antisense *PHYA* plants with reduced expression of endogenous *PHYA*. The present inventors thus make available novel transgenic plants with improved rooting capacity, as well as a method for their production, as defined in the attached claims.

Brief description of the drawings

The invention will be described in closer detail in the following description and and non-limiting examples, with reference to the attached drawings, in which

Figure 1 consists of two photographs, showing the apical part of *AsPttPHYA* line 7 and wild type hybrid aspen grown in tissue culture, the former showing the conditional phenotype of short internodes and bud formation at the apex;

Figure 2 is a photograph showing roots from *AsPttPHYA* line 7 and wild type hybrid aspen grown in tissue culture, the former clearly showing increased root growth;

Figure 3 shows a Northern blot analysis of early expanding leaf tissue showing the levels of *PHYA* transcript in wild type and transformed lines as detected by an antisense *PttPHYA* riboprobe. Total RNA, 48 µg, was loaded from samples of all but line 7, and 38 µg of a corresponding wild type sample (indicated by*). As a loading control, the ethidium bromide staining shows rRNA intensity.

Figure 4 shows the cumulative shoot elongation (cm) showing growth of wild type and *AsPttPHYA* lines grown in long photoperiod (18 h);

Figures 5A and B being Northern blot analyses show the WT levels of *PttPHYA* transcript in different tissues, and photoperiods. Eight µg of poly A+ RNA was loaded for each sample. 5A) shows the expression under LD in, left to right: young roots (YR), mature non-expanding internode (MI), late expanding internode (LI), early expanding internode (EI) and apex (A). 5B) shows the expression under LD is compared to SD for young roots (YR), late expanding

internode (LI) and apex (A). For comparison, levels of the endogenous ubiquitin genes, *PttUBQ1* or *PttUBQ2* are also shown.

Description

Before the present method for improving the rooting capacity of plants is disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein – with the exception of the sequence according to SEQ. ID. NO. 1 and sequences homologuous therewith – as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

Propagation of woody species by rooting of cuttings or via *in vitro* propagation is in many cases very difficult. The physiological and molecular reasons for some species being difficult to root are not known, but there are possibilities that specific "root induction" genes are more inhibited in some species than in others. The communication between root and shoot is also believed to be of importance as there is an old concept of root growth versus shoot growth. The present inventors have investigated the role of phyA in the process of root growth, and shown for the first time that the level of *PHYA* is of importance for rooting ability and biomass. This is of considerable practical importance as it makes it possible to speed-up the multiplication of important genetic plant material, and this is achieved merely by genetically modifying the expression of one gene. The growth results also show that the overall growth during the growth season (i.e. long days) was not negatively affected. Rather the survival is higher as the increased rooting capacity also increases the survival after potting. The increased sensitivity to short days does not seem to have any negative effect on the total growth.

The present invention makes available a plant cell comprising a polynucleotide sequence reducing the expression of a photoreceptor gene, and a plant comprising such a plant cell, wherein the expression of endogenous phytochrome A (*PHYA*) is reduced.

It has been shown by the present inventors that the plant according to the invention exhibits improved rooting capacity as compared to the wild type of said plant. It has further been

shown that the plant according to the invention exhibits improved survival after potting as compared to the wild type of said plant.

It is noted, that the prior art has indicated that phytochromes are present in all tissues of plants, including in the roots, and that they may have a functional role e.g. in controlling cell morphology. It is not unexpected that root cell morphology should also be influenced by phytochromes, as it is just as important to a root to be able to detect light and react to it. It is however very surprising that by lowering the amount of one phytochorome, namely phyA, should lead to a dramatic increase in rooting and rooting ability.

According to an embodiment of the invention, the polynucleotide sequence is a sequence capable of reducing the expression of a photoreceptor gene influencing photomorphogenetic development, and in particular an antisense construct corresponding to such a photoreceptor gene. According to a preferred embodiment, the polynucleotide sequence comprises a sequence capable of reducing the expression of a sequence substantially similar or substantially homologous to SEQ ID NO:1, such as a sequence hybridising therewith under stringent conditions, or in particular an antisense sequence of the nucleic acid sequence of SEQ ID NO:1.

According to the invention, the plant is a woody plant belonging to a tree species, and in particular a plant chosen among aspen, spruce, pine, birch, oak, and eucalyptus.

Further, according to the invention, the plant is a decorative plant.

The invention also comprises propagating material of a plant according to any one of the above specifications, for example a seed of such a plant.

The invention further makes available a method for improving the rooting capacity of plants, comprising the steps of

a) transforming a plant cell with a polynucleotide sequence causing reduced expression of endogenous phytochrome A (*PHYA*);

b) regenerating the plant cell into a plant; and

c) selecting a plant with improved rooting capacity compared to the wild type of the same plant.

According to the inventive method, the polynucleotide sequence comprises a sequence capable of reducing the expression of a sequence substantially similar or substantially homologuous to SEQ ID NO:1, or a sequence hybridising therewith under stringent conditions. In particular, the polynucleotide sequence comprises the antisense sequence corresponding to SEQ ID NO:1.

The present invention makes available a method for improving the survival after potting of plants, comprising the steps of

a) transforming a plant cell with a polynucleotide sequence causing reduced expression of endogenous phytochrome A (*PHYA*);

b) regenerating the plant cell into a plant; and

c) selecting a plant with improved survival after potting capacity compared to the wild type of the same plant.

According to the inventive method, the polynucleotide sequence comprises a sequence capable of reducing the expression of a sequence substantially similar or substantially homologuos to SEQ ID NO:1, or a sequence hybridising therewith under stringent conditions. In particular, the polynucleotide sequence comprises the antisense sequence corresponding to SEQ ID NO:1.

The method will be described in closer detail in the following non-limiting examples:

Examples

1.1. Regeneration of antisense PHYA hybrid aspen

The isolation of a full-length cDNA clone for hybrid aspen, *PHYA*, has been described earlier (Eriksson, M. and Moritz, T. (1997). Isolation of a cDNA Encoding a Phytochrome A (Accession No. AJ001318) from *Populus tremula* x *tremuloides*. Plant Gene Register # PGR97-186. *Plant Physiol.* **115**, 1731.). For the antisense construct a full-length cDNA fragment was cloned in reverse direction into the *Bam HI* site behind the 35S CaMV promoter of the vector pPCV702.kana. The construct was introduced by *Agrobacterium tumefaciens* mediated transformation into hybrid aspen (Nilsson, O., Aldén, T., Sitbon, F., Little, C. H. A., Chalupka, V., Sandberg, G. and Olsson, O. (1992). Spatial pattern of cauliflower mosaic virus

35S promoter-luceferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. *Transgenic Res.* 1, 209-220). Seven independently transformed lines (*AsPttPHYA*-plants) were regenerated. All but line 5, which showed a WT phenotype, were shown in tissue culture to have a phenotype featuring compact growth, apical bud formation (Fig. 1), and increased root growth (Fig. 2).

To investigate the antisense effect on the RNA level, leaves were harvested in LD conditions in the middle of the photoperiod. Leaves were chosen for this purpose, despite the low expression of *PttPHYA* in their tissues, since they are probably the site of photoperiodic sensing in many woody species. The analysis showed that the expression of the *PHYA* transcript was moderately reduced in the transformed lines, and there was no detectable down-regulation in line 5 compared to WT (Fig. 3). All transgenic lines were shown to express the antisense transcripts (data not shown).

1.2. Down-regulation of PHYA affects the formation of roots in tissue culture

The rooting capacity *in vitro* was increased in the lines with reduced *PHYA* levels (Fig. 2; Table 1). The root weight expressed as fresh weight was increased between 74 and 114 %. The increased root weight also improved the survival after potting, where 100% of antisense lines survived, but approx. 80 % of wild type survived.

Table 1. Increased root growth in *AsPttPHYA* lines 1, 2 and 7

Plants were grown in tissue culture and number of plants per line = 3. Weight is expressed as mg fresh weight ± standard deviation.

Wild type	AsPttPHYA-1	AsPttPHYA-2	AsPttPHYA-7
14,1±4,3	24,3±7,3	27,5±9,2	30,2±6,7

1.3. Down-regulation of PHYA affects SD induced cessation of apical growth but not growth under LD

The effect of different photoperiods on growth of the transformants was first tested with all seven regenerated lines in a growth experiment. In LD (18 h) conditions the transgenic lines showed slightly increased elongation growth compared to the WT plants (Fig. 4). This was

probably due to their increased root biomass in tissue culture, which might promote early establishment after potting in soil. However, in SD conditions (10 h) all transgenic lines but line 5 showed increased sensitivity towards a short photoperiod, resulting in faster cessation of growth (Table 2).

Table 2. Sensitivity to short photoperiods in WT and transformed lines

The values from experiment (exp.) 1 show the cumulative height growth when the growth was completely stopped (n=3-5). The values from exp. 2 show the number of days to a visible bud is formed in short photoperiod (n=7-11). Statistical significance differences as compared to WT are indicated at the 1 % (*) and 5 % (**) level, Fisher' Protected Least Significant Difference (PLSD).

Line	Cumulative growth (cm ± STDEV) after 28 days in 10 h photoperiod (Exp. 1)	Days (±STDEV) to bud set in 15 h photoperiod (Exp. 2)
WT	32,5 ± 3,0	40,4 ± 2,5
As-1	28,6 ± 4,0**	35,2 ± 3,1*
As-2	29,0 ± 3,5**	34,9 ± 2,9*
As-4	27,3 ± 2,6*	-
As-5	30,0 ± 5,8	-
As-7	26,3 ± 4,7*	-
As-8	29,0 ± 2,6**	-
As-9	29,3 ± 5,7	33,6 ± 4,7*

1.4. Expression of PHYA is both spatially and photoperiodically regulated

The expression pattern of the *PHYA* gene in different tissues of hybrid aspen was investigated. Plants were grown under a long photoperiod, and poly $(A)^+$ RNA was extracted from young roots, the apex, early expanding, late expanding and mature non-expanding leaf blades, and internodes. Northern blot analysis showed that the highest levels of expression of the transcript in LD (18 h) occur in root tissue and in mature non-expanding internodes (Fig. 5 A). The expression of *PHYA* in the apical region, (Fig. 5 A) leaves and petioles was weak (data not shown), and no difference in expression was observed between different leaf positions.

To investigate possible differences in *PHYA* transcript levels under different photoperiods, plants grown under long photoperiods were transferred to a short photoperiod (10 h). Plants were harvested in the middle of the photoperiod both before the transfer and after four days of the short photoperiod. Results of the subsequent northern analysis show that there was a clear up-regulation of *PHYA* transcripts in the apex and elongating internode tissues, but a down-regulation in young root tissue, causing the strongest expression of *PHYA* to occur in internode tissue, in marked contrast to the expression patterns under LD (Fig 5B). Furthermore the expression levels in leaves were also very low compared to other tissues in SD (data not shown).

1.5.Antisense PHYA plants suggests that phyA has an important role in root growth

The root biomass was dramatically increased in lines with reduced levels of *PHYA* (Fig. 2, Table 1). This shows that phyA might be an indirect regulator of root formation. There is a strong expression of *PHYA* in roots of hybrid aspen (Fig. 5a). This fits well with results from promoter-GUS studies in *Arabidopsis* and tobacco, quantitative studies of mRNA in tomato and protein studies in oat. Although the phyA protein has been shown to be both present and functional in root tissue and has been suggested to be involved in gravitropic sensing, there is still very little data on phytochrome effects on root physiology. The present inventors, however, show for the first time that changes by genetic modification can significantly affect the root growth.

1.6. Photoperiodic response of antisense PHYA plants suggests phyA also has a role in the photoperiodic control of apical growth cessation

All transformed lines apart from line 5 showed a conditional dwarfed phenotype with apical bud formation in tissue culture. This is similar to tissue propagated *Populus tremula* in the same environment (data not shown). The *P. tremula* clone tested is a local ecotype with a longer critical daylength than the hybrid aspen T89 clone used. This indicates that the light conditions in the tissue culture, with respect to daylength and light quality, may have been unfavourable for apical shoot growth of the antisense *PttPHYA* plants and *P. tremula*. When transferred from tissue culture to LD conditions in the green house or growth chamber the antisense plants started to elongate like WT plants (Fig. 3). This shows that the dwarfed phenotype in tissue culture is not persistent, and the increased root biomass actually is a help during the establishment after potting on soil.

PttPHYA antisense plants show higher sensitivity to short photoperiods, with earlier and faster cessation of apical growth than WT (Table 2). In the previous work with hybrid aspen expressing the oat *PHYA*, the overexpression caused insensitivity to short days. Both present and previous results suggest, therefore, that phyA mediates photoperiodic control of apical growth cessation in *Populus tremula* x *P. tremuloides*. It can be argued that in general phyB is involved in short day induced responses. It has been shown, for instance in the SDP potato that antisense *PHYB* expression will lead to loss of the photoperiodic control of tuberisation. However, recent data from potato have shown that phyA is also important in the photoperiodic regulation of tuber formation (Yanovsky, M. J., Izaguirre, M., Wagmaister, J. A., Gatz, C., Jackson, S. D., Thomas, B. and Casal, J. J. (2000). Phytochrome A resets the circadian clock and delays tuber formation under long days in potato. *Plant J.* 23, 223-232). Tuber formation is accelerated in transgenic potato with reduced *PHYA* expression compared to control plants when the day is extended with far red plus red light. The results show that phyA delay short day responses such as tuber formation in potato under LDs.

Further evidence that the induction of dormancy in the hybrid aspen is not mediated via phyB is that SD with end-of-day far red (EOD FR) treatments does not inhibit dormancy induction. EOD FR treatment has been suggested to be mediated primarily by light stable phytochromes, such as phyB. Flowering in SDPs is also inhibited when plants are grown in inductive SDs and then subjected to EOD FR treatments. Data gathered by the present inventors therefore suggests that the induction of dormancy in hybrid aspen is a LDP response, in which LD inhibits growth cessation. Thus, phyA is likely to inhibit bud set, by sustaining growth in a similar fashion to the flower promoting effect seen in *Arabidopsis* and pea. This would also explain the daylength independent growth seen in the hybrid aspen overexpressing oat *PHYA*. Furthermore, there is evidence suggesting that in LDPs, such as *Arabidopsis* and pea, phyA plays an important role in the perception of daylength. Earlier photobiological experiments performed using black cottonwood (*Populus trichocarpa*), suggesting that the black cottonwood induction of dormancy is a SD sensitive response, are not supported by the experimental data from hybrid aspen by the present inventors. There are, however, examples of species in which LDP, SDP and day neutral plant (DNP) varieties occur.

1.7. The PHYA antisense inhibition is specific

It could be argued that other phytochromes could also be affected directly or indirectly by the antisense inhibition and thus cause the observed changes in sensitivity to SDs. A direct effect

is unlikely, however, since the homology between different phytochromes in general is not high. *Arabidopsis* PHYA and PHYB show 52% homology at the amino acid level (Clack, T., Mathews, S. and Sharrock, R. A. (1994). The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: The sequences and expression of *PHYD* and *PHYE*. *Plant Mol. Biol.* 25, 413-427) and the more closely related PHYB and PHYD show 80% homology. In the deciduous tree species *Populus trichocarpa*, where the phytochrome gene family has been characterised, one *PHYA* locus and two *PHYB* loci have been identified. The homology between the partial *PHYA* and *PHYB* cDNA clones is about 55%, but the homology between the hybrid aspen *PHYA* clone used in the present examples and *Populus trichocarpa PHYA* is higher than 98%. The low degree of conservation between *Populus* PHYA and PHYB suggest that with the antisense construct of *PttPHYA* the inhibition will be restricted to *PHYA*. Further, phenotypic, evidence for specific *PHYA* inhibition is provided by the WT-like growth in LD, which markedly differs from the slender phenotype that has been observed in antisense *PHYB* potato plants and is also characteristic of the *phyB* mutant of e.g. *Arabidopsis*.

1.8. PHYA expression is both spatially and photoperiodically regulated

The importance of phytochrome A in photoperiodically growth-regulated trees has now been shown both in the present investigation and in the previous work with hybrid aspen expressing the oat *PHYA* . Therefore the present inventors also conducted a spatial and photoperiodic characterisation of *PttPHYA* in hybrid aspen. As has been shown in other species such as *Arabidopsis*, tobacco and tomato, expression of *PHYA* was found in all tissues examined, including root, internode, apical tissues, (Fig. 5A) leaf and petiole (data not shown). The gradual increase in *PHYA* transcript levels from the apex to the lower internodes and roots of hybrid aspen suggests a light-dependent down-regulation of *PHYA* expression occurs, similar to that observed in studies of *Arabidopsis PHYA* and *Pharbitis nil*.

This observation in hybrid aspen may be the results of the gradual decrease in light exposure from the apex to the root. No gradient was found in the leaves, but they were all exposed to about the same light intensity (data not shown). It should be emphasised that under SD conditions the expression of *PHYA* was different from those under LD conditions (Fig. 5B). The expression of *PHYA* was up-regulated in the internode and apex upon transfer to SD, but down-regulated in root tissue.

Although little is known about *PHY* RNA levels in relation to photoperiod, there is now evidence that phytochrome B gene transcription is controlled by the circadian clock. It is tempting to suggest that the *PHYA* level is controlled by the circadian clock, and the earlier light-on signal perceived in LD grown plants may result in an altered circadian rhythm compared to the SD grown plants. However, this remains to be investigated. So far evidence for a circadian regulation of *PHYA* expression has not been reported. The up-regulation of *PHYA* in the internode and apex upon transfer to SD may also be due to synthesis of *PHYA* RNA occurring during the dark period. Under SDs the time from dark to the sampling point (the middle of the photoperiod) is shorter than in LDs, so there is less potential for light-induced inhibition of *PHYA* transcription.

2. Materials and methods

2.1. Antisense vector construction

The antisense *PttPHYA* vector was constructed using the *PttPHYA* full length cDNA clone (accession number AJ001318) described in Eriksson and Moritz (1997) which is inserted between the *Eco RI* and *Xho* I sites in the phagemid pBK-CMV (Stratagene, La Jolla, CA). The construct was cut with *Xho* I, filled in with Klenow, and *Bam HI* linked. The insert was retrieved as a *Bam HI* fragment, and ligated into the *Bam HI* site of pPCV702.kana (Koncz, C. and Schell, J. (1986). The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimaeric genes carried by a novel type of binary vector. *Mol. Gen. Genet.* **204**, 383-396.). Constructs carrying the cDNA in antisense orientation were identified by enzymatic digestion and further confirmed by sequencing from a specific primer complementary to the 35S CaMV promoter binding approximately 110 bp upstream from the *Bam HI* cloning site; 5'-GCAAGTGGATTGATGTG-3'. The resulting construct is denoted *AsPttPHYA* and includes the full length *PttPHYA* cDNA in an inverted position driven from the CaMV 35S promoter, and it was further used to transform hybrid aspen.

2.2. Plant Transformation

Transformation of *AsPttPHYA* into hybrid aspen (*Populus tremula* L. x *P. tremuloides* Michx.) and regeneration was done essentially as previously described (Nilsson, O., Aldén, T., Sitbon, F., Little, C. H. A., Chalupka, V., Sandberg, G. and Olsson, O. (1992). Spatial pattern of cauliflower mosaic virus 35S promoter-luceferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. *Transgenic Res.* 1, 209-220). The seven independent lines produced were all multiplied by cuttings and rooted *in*

vitro on half-strength MS medium containing minerals and vitamins. Wild type control plants were multiplied in tissue culture in parallel with the transgenic plants.

2.3. RNA isolation and gel blot analysis

Total RNA from the seven independent hybrid aspen lines (lines 1, 2 4, 5, 7, 8, and 9) transformed with the *AsPttPHYA* construct and WT was obtained using a CTAB method for RNA extraction (Chang, S., Puryear, J. and Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* **11**, 113-116.) Either 48 µg or 38 µg (see figure text) total RNA from each sample was loaded onto formaldehyde agarose gels and separated according to Sambrook *et al.*, (1989) [Sambrook, J., Fritsch, E. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* 2nd edn. Cold Spring Harbor : Cold Spring Harbor Laboratory Press] followed by capillary blotting onto nylon membranes (Hybond N, Amersham Pharmacia, Little Chalfont, UK) and cross linking by UV light (SpectroLinker XL 1000, Spectronics Corporation, Westbury, NY).

As a template for generating riboprobes a PCR fragment from the *PttPHYA* (position 2630 to 3047 bp), cloned between the T7 and T3 polymerase promoters of the puc 19 derived pT7/T3α-19 was used. Sense and antisense riboprobes were synthesised using a Strip EZ™ RNA T3 kit (Ambion Inc, Austin, TX, USA), transcription from the T7 promoter was carried out substituting the T3 polymerase mix with T7 RNA polymerase, and the RNA inhibitor RNAGuard (Amersham Pharmacia Biotech, Uppsala, Sweden) at recommended concentrations. Hybridisation was carried out at 65°C as described by Ait-Ali *et al.*, (Ait-Ali, T., Frances, S., Weller, J. L., Reid, J. B., Kendrick, R. E. and Kamiya, Y. (1999). Regulation of gibberellin 20-oxidase and gibberellin 3 beta-hydroxylase transcript accumulation during de-etiolation of pea seedlings. *Plant Physiol.* **121**, 783-791.), washing the membranes at the same temperature for five minutes in 2 x SSPE, 0.1 % (w/v) SDS and then two times five and two times fifteen minutes in 0.1 x SSPE, 0.1 % (w/v) SDS, before exposing them to Phosphoimager screens (Molecular Imager GS-525, Bio-Rad Laboratories, Hercules, CA, USA). Between hybridisations the Strip EZ™ RNA T3 kit (Ambion) was used to remove the probe.

2.4. Poly A+ extraction and analysis

Total RNA extraction (as above) was followed by poly $(A)^+$ purification on oligo (dT)-cellulose type 7 (Amersham Pharmacia Biotech) according to the manufacturer's

recommendations, except that LiCl was substituted for NaCl. Eight µg of poly A+ RNA from each sample was loaded and blotted as described above. The blots were probed with purified alpha -[^{32}P] dCTP (6000Ci/mmol; Amersham) labelled DNA fragments, which were hybridised, washed and exposed to Phosphoimager screens as previously described (Eriksson, M. E., Israelsson, M., Olsson, O. and Moritz, T. (2000). Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat. Biotechnol.* **18**, 784-788.). Northern blots were probed with a 1171 bp *Eco RI/ Xba* I *PttPHYA* fragment from its 3'-end, stripped with boiling hot 0.1 x SSPE, in 0.1% (w/v) SDS and reprobed with the ubiquitin-like ESTs, A046p07u (*PttUBQ1*) or A081p57u (*PttUBQ2*) (Sterky, F., Regan, S., Karlsson, J., Hertzberg, M., Rohde, A., Holmberg, A., Amini, B., Bhalerao, R., Larsson, M., Villarroel, R. et al. (1998). Gene discovery in the wood-forming tissues of poplar: Analysis of 5,692 expressed sequence tags. *Proc. Natl Acad. Sci. USA*, 95, 13330-13335.).

2.5. Growth experiments and sampling

Complete root tissue from hybrid aspen plant grown in tissue culture were sampled and weighted on a Mettler AT20 balance (±10+ µg).

Hybrid aspen plants for growth experiments were potted in fertilised peat and cultivated in the greenhouse until the plants were stabilised and growing well. The plants were grown under an 18 h photoperiod in the greenhouse, with day and night temperatures of 20°C and 15°C, respectively. Photoperiodic experiments were carried out in a growth chamber at 18°C and a relative humidity of 90%. The photoperiod in LD conditions was 16 or 18 h long, with a 10 h main light period, in which the 400–750 nm photon flux density was set at 175 µmol m^{-2} s^{-1} (Osram Power Staw HQI-TS 400 W/D lamps, Osram, Germany). Daylength extensions of 6 or 8 h were given in LD using low-intensity light (20 μmol^{-2} s^{-1}) before or after the main light period. The light qualities in the SD conditions were the same as for the LD main period, with 10 h photoperiods in experiment (exp.) 1 and 15 h in exp. 2. Plants were watered daily, and repotted and fertilised with a complete nutrient solution (SuperbaS, Supra Hydro AB, Landskrona, Sweden) once a week.

In exp. 1, between three and five plants of each of the seven transgenic lines and the WT, were screened at 18°C for their growth responses under LD and SD conditions, with 18 h and 10 h photoperiods, respectively. Cumulative stem elongation was recorded every 3rd to 5th day

over a period of 17 days under LD and 28 days under SD. In exp. 2, three lines (lines 1, 2 and 9) were selected for further experiments. Together with the WT, these plants (7 to 11 per line) were tested over a period of 30 days under LD (18 and 16 h) and 46 SD (15 h) photoperiods. Materials for RNA analysis were harvested in exp. 1 from early expanding leaves of plants growing in LDs. Samples were harvested in the middle of the main photoperiod and leaves were taken and pooled from two plants per line.

For the characterisation of the endogenous *PttPHYA* RNA expression, 11 weeks old wild type plants were first grown under LD conditions (18 h), two plants were sampled and the remaining two plants were transferred to SD (10 h) and harvested after four more days. All samples were harvested in the middle of the main photoperiod. Samples were collected from plants 1.7 – 1.9 m tall, from the apices and internodes 9, 27 and 68, together with adjacent leaves and petioles as well as actively growing roots. The sampling positions, internode 9, 27 and 68 corresponded to tissues being early expanding, late expanding and mature non-expanding, respectively. Tissues from two plants were harvested and pooled for each sample. The 'first internode' was defined as the first internode below a leaf at least 1 cm long.

The results show that not only is the rooting capacity improved, but also the overall survival of the transgenic plants. This is an additional advantage of the present invention. It is also advantageous that this surprising result is achieved after regulating the expression of one single gene. The inventive method is comparatively simple and easy to perform also in large scale plant breeding.

It is also an advantage that the antisense inhibition according to the invention is specific and was not observed to cause changes in SD sensitivity.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention as set forth in the claims appended hereto.

Claims

1. A plant cell comprising a polynucleotide sequence reducing the expression of a photoreceptor gene which influences photomorphogenetic development by reducing the expression of endogenous phytochrome A (*PHYA*).

2. A plant, the cells of said plant comprising a plant cell according to claim 1, wherein the plant exhibits improved rooting capacity as compared to the wild type of said plant.

3. The plant according to claim 2, wherein the plant exhibits improved survival after potting as compared to the wild type of said plant.

4. The plant according to claim 2, wherein the polynucleotide sequence is an antisense construct corresponding to a photoreceptor gene.

5. The plant according to claim 2, wherein the polynucleotide sequence is a sequence capable of reducing the expression of a sequence according to SEQ ID NO: 1 or a sequence substantially homologous therewith.

6. The plant according to claim 2, wherein the polynucleotide sequence comprises the antisense sequence corresponding to sequence of SEQ ID NO:1.

7. The plant according to any one of the claims above, wherein the plant is a plant belonging to a woody plant species.

8. The plant according to any one of the claims above, wherein the plant is one of aspen, spruce, pine, birch, oak, and eucalyptus.

9. The plant according to any one of the claims above, wherein the plant is a decorative plant.

10. Propagating material of a plant according to any one of claims 2 – 9.

11. A seed of the plant according to any one of the claims 2 – 9.

12. A method for improving the rooting capacity of plants, comprising the steps of

d) transforming a plant cell with a polynucleotide sequence causing reduced expression of endogenous phytochrome A (*PHYA*);

e) regenerating the plant cell into a plant; and

f) selecting a plant with improved rooting capacity compared to the wild type of the same plant.

13. The method according to claim 12, wherein the polynucleotide sequence is a sequence capable of reducing the expression of a sequence according to SEQ ID NO:1 or a sequence substantially homologous therewith.

14. The plant according to claim 12, wherein the polynucleotide sequence comprises the antisense sequence corresponding to the sequence of SEQ ID NO:1.

15. A method for improving the survival after potting of plants, comprising the steps of

d) transforming a plant cell with a polynucleotide sequence causing reduced expression of endogenous phytochrome A (*PHYA*);

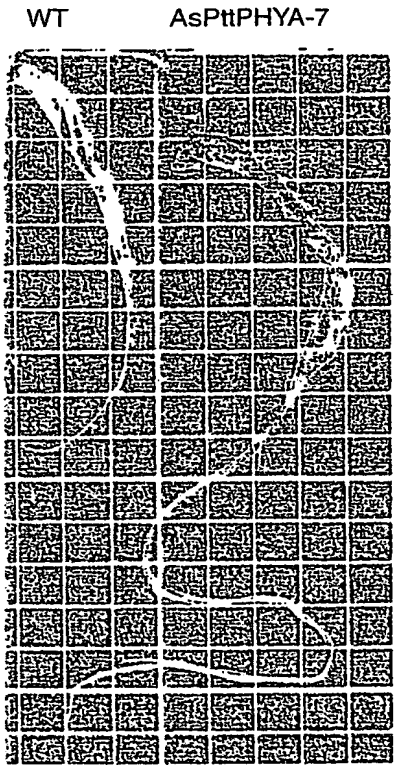
e) regenerating the plant cell into a plant; and

f) selecting a plant with improved survival after potting capacity compared to the wild type of the same plant.

16. The method according to claim 15, wherein the polynucleotide sequence is a sequence capable of reducing the expression of a sequence according to SEQ ID NO:1 or substantially homologous therewith.

17. The method according to claim 15, wherein the polynucleotide sequence comprises the antisense sequence corresponding to the sequence of SEQ ID NO:1.

Fig. 1



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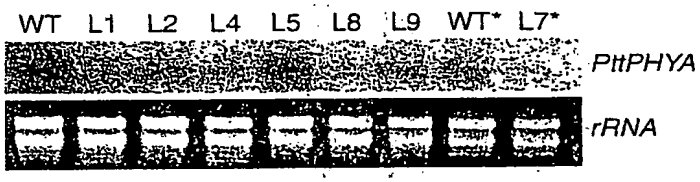

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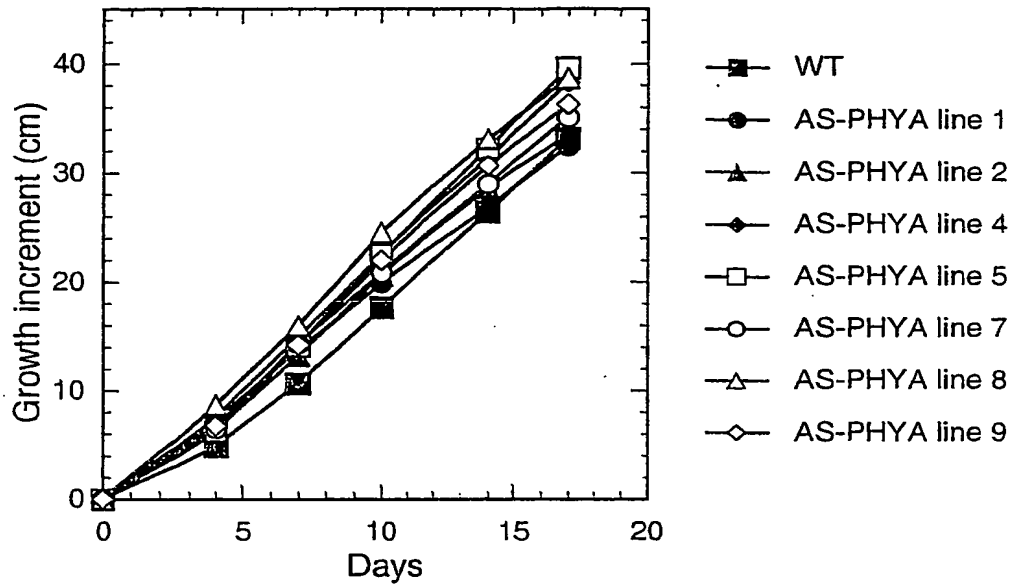
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10
20
30
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15
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AS-PHYA line 9

Fig. 4

Fig. 5 A

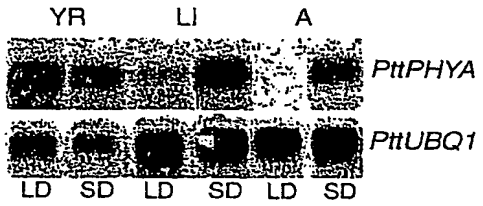

Fig. 5 B

SEQUENCE LISTING

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MORITZ, Thomas
ERIKSSON, Maria

<120> Transgenic plants exhibiting improved rooting and methods for their production

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<141>

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<151> 2000-10-25

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<301> ERIKSSON, Maria E.
MORITZ, Thomas
<302> Isolation of a cDNA encoding a phytochrome A (Accession No. AJ001318) from Populus tremula x tremuloides
<303> Plant Physiol.
<304> 115
<306> 1731-
<307> 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 01/02245

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A01H 1/00, A01H 5/08, C12N 5/29
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Plant Physiol, Volume 109, 1995, Arnd G. Heyer et al: "Function of Phytochrome A in Potato Plant as Related through the Study of Transgenic Plants", pages 53-61, see specially page 54 "Construction of transgenic plant lines" --	1-11
A	Eriksson M.E., et al: "Isolation of a cDNA Encoding a Phytochrome A (Acc.no. AJ001318) from Populus tremula x tremuloides. (PGR97-186), Plant Physiol. 115:1731-1731(1997) & Database EMBL Accession no. PTXPTPHYA, 4 December 1997 --	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 01/02245

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Plant Molecular Biology, Volume 18, 1992, Arnd Heyer et al: "Isolation and characterization of cDNA-clone coding for potato type A phytochrome", pages 535-544 -- --------	1-17

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(71) Applicant *(for all designated States except US)*: **SWETREEGENOMICS AB** [SE/SE]; P.O. Box 7984, S-907 19 Umeå (SE).

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(74) Agent: **HOLMBERG, Martin**; Bergenstråhle & Lindvall AB, P.O. Box 17704, S-118 93 Stockholm (SE).

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(54) Title: TRANSGENIC PLANTS WITH REDUCED EXPRESSION OF ENDOGENOUS PHYTOCHROME A, EXHIBITING IMPROVED ROOTING, AND METHODS FOR THEIR PRODUCTION

(57) Abstract: A method for improving the rooting capacity of plants by downregulating the phytochrome A is disclosed, as well as transgenic plants having improved rooting capacity. Also the overall survival after potting capacity is improved in the plants produced according to this method.

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INTERNATIONAL SEARCH REPORT

International application No.
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B. FIELDS SEARCHED

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Plant Molecular Biology, Volume 18, 1992, Arnd Heyer et al: "Isolation and characterization of cDNA-clone coding for potato type A phytochrome", pages 535-544	1-17

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(54) Title: TRANSGENIC PLANTS WITH REDUCED EXPRESSION OF ENDOGENOUS PHYTOCHROME A, EXHIBITING IMPROVED ROOTING, AND METHODS FOR THEIR PRODUCTION

(57) Abstract: A method for improving the rooting capacity of plants by downregulating the phytochrome A is disclosed, as well as transgenic plants having improved rooting capacity. Also the overall survival after potting capacity is improved in the plants produced according to this method.